Abstract. Hepatitis C virus (HCV) nonstructural protein 5A (NS5A) modulates cellular apoptosis, which is involved in the occurrence and development of liver cancer. The mechanisms of apoptosis inhibition by NS5A in liver cancer cells remains unclear. Beclin 1, which functions upstream of autophagosome formation, is upregulated by NS5A. Autophagy, an evolutionarily conserved catabolic process, has a crucial role in tumor initiation and progression. Autophagy was blocked by inhibitors 3-methyladenine and chloroquine, or via knockdown of Beclin 1. Flow cytometric analysis and western blotting were used to detect apoptosis. It was found that inhibition of autophagy attenuated the NS5A-mediated apoptosis inhibition of HepG2 cells. Furthermore, it was confirmed that Beclin 1 expression by NS5A was involved in the negative regulation of starvation-induced liver cancer apoptosis, which was accompanied by reduced p53 and apoptosis regulator Bax expression, as well as decreased caspase-3/-7 activation. Therefore, inhibition of autophagy may be promising therapeutic strategy in the treatment of HCV-associated liver cancer.

Introduction

Persistent Hepatitis C virus (HCV) infection may result in liver cancer. In 2011, 130-170 million people were infected with HCV worldwide. Liver cancer develops in 1-4% patients with HCV-induced cirrhosis annually (1). Understanding the mechanisms that underlie the development of HCV into liver cancer is important for HCV-associated liver cancer treatment. HCV nonstructural 5A (NS5A) encodes a 447 amino acid phosphoprotein (2). This protein serves as a transcriptional activator of cell growth. It interacts with other proteins and has a crucial role in hepatocarcinogenesis (3). According to a previous study, NS5A inhibits cell apoptosis in vivo and in vitro (4). Peng et al (5) reported that NS5A decreases caspase-3 cleavage (5). Lan et al (2) demonstrated that HCV NS5A suppresses p53-mediated transcriptional transactivation and apoptosis during HCV infection, contributing to hepatocarcinogenesis. In addition, NS5A significantly increases the expression of inducible nitric oxide synthase, cyclin D1 and nuclear factor-κB, but decreases p53 protein expression in HepG2 cells (6). By regulating the expression of several genes in host liver cells, NS5A also induces cellular proliferation, and influences the curative effect of interferon (7).

Autophagy provides energy to tumor cells for survival and metabolic reprogramming, in order to accommodate rapid cell growth and proliferation (8). Increasing amounts of evidence indicate that autophagy is induced by a number of stressors in tumor cells, such as starvation, growth factor deprivation, hypoxia, damage stimulation and therapeutic drugs, and is an important survival mechanism in response to cellular stress (9). Beclin 1, the mammalian counterpart of the yeast Atg6 gene, is an essential protein in autophagy (10). Using cDNA microarray screens and northern blot analysis, a previous study showed that Beclin 1 is upregulated in liver cancer tissues (11). In addition, Beclin 1 gene deletion results in tumor cell apoptosis, specifically in hypoxic regions (12). Liu et al (13) reported that Beclin 1 gene deletion by either RNA interference or the autophagy inhibitor 3-methyladenine (MA) significantly enhances melatonin-induced apoptosis in mouse hepatoma H22 cells. Guo et al (14) found that autophagy inhibition significantly increases liver cancer cell apoptosis during nutrient starvation or hypoxia in vitro. NS5A upregulates Beclin 1 mRNA and protein expression in a HCV NS5A-transactivated protein 9 (NS5ATP9)-dependent manner (15). In addition, NS5A could induce autophagy in a Beclin 1-dependent manner. Thus, the present study hypothesized that NS5A inhibits apoptosis by inducing Beclin 1-dependent autophagy.
Materials and methods

Construction of plasmids. pcDNA3.1/myc-His(-)-NS5A (pNS5A) was constructed as previously described (15). The pNS5A was amplified using the primer set 5’-GATATCATGAAGGGTCTAAGACGTCTC-3’ and 5’-GGAATCCTCATTTGTTAAAAATTTGAGG-3’. Total RNA from L02 cells was prepared using a total RNA kit (R6834; Omega, Norcross, GA, USA) according to the manufacturer’s instructions. mRNA was reverse transcribed into cDNA using a PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). The RT temperature protocol was 37°C for 15 min and 85°C for 5 sec. The amplified product was digested with EcoRV and BamHI (Takara Biotechnology Co., Ltd.) and inserted into the vector pcDNA3.1/myc-His(-).

siRNA oligonucleotides. Beclin 1 small interfering RNA ([siRNA] cat. no. sc-29797) and negative control siRNA (cat. no. sc-37007) were purchased from Santa Cruz Biotechnology Co., Ltd. (Dallas, TX, USA).

Cell culture and transfection. Hepatoblastoma HepG2 cells were obtained from the Chinese Academy of Science Cell Bank (Shanghai, China) and cultured in Dulbecco's modified Eagle’s medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin G and 100 µg/ml streptomycin in a humidified incubator at 37°C in a 5% CO₂ atmosphere. Cells were cultured to 60-80% confluence and transiently transfected with 50 nM Beclin 1 siRNA, 50 nM siRNA negative control for 48 -72 h, 2 µg pNS5A or 2 µg plasmid control for 48 -72 h using Polyplus transfection reagent (Polyplus-transfection SA, Illkirch, France) according to the manufacturer’s protocols.

Starvation was induced by amino acid deprivation in Earle’s Balanced Salt Solution (EBSS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at different time points (24-48 h). 3-methyladenine 10 mM (3-MA; Sigma-Aldrich, St. Louis, MO, USA) and chloroquine 10 µM (CQ, InvivoGen, Shatin, Hong Kong, tlrl-chq) were added to the medium for 24 h to block autophagy.

Western blotting. Protein was extracted from HepG2 cells using lysis buffer containing a protease inhibitor cocktail (Cell Signaling Technology, Inc., Danvers, MA, USA). Equal amounts of protein (40-60 µg/lane) were separated on 12% Bis-Tris Gel/MOPS (cat. no. NP0034; Invitrogen; Thermo Fisher Scientific, Inc.) and transferred to polyvinylidene difluoride membranes by electroblotting. Following blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies against LC3B (cat. no. 3495; Cell Signaling Technology, Inc.), GAPDH (cat. no. 5174; Cell Signaling Technology, Inc.), Beclin 1 (cat. no. 3495; Cell Signaling Technology, Inc.), p53 (cat. no sc-126; Santa Cruz Biotechnology Co., Ltd.), apoptosis regulator BAX (Bax; cat. no. sc-493; Santa Cruz Biotechnology Co., Ltd.), cleaved caspase-3 (cat. no. 9579s; Cell Signaling Technology, Inc.) and anti-His (cat. no. sc-803; Santa Cruz Biotechnology Co., Ltd.). Protein bands were detected by an enhanced chemiluminescence system (cat. no. 32209; Thermo Fisher Scientific, Inc.). Western blotting data were quantified using Bio1D software (S:11.640150; Vilber Lourmat, Marne-la-Vallée, France).

Flow cytometry. Following transfection and starvation at different times, cells were harvested and washed two times with cold BioLegend cell staining buffer (cat. no. 420201; BioLegend, Inc., San Diego, CA, USA), and then resuspended in the Annexin V binding buffer (cat. no. 422201; BioLegend, Inc.) at a concentration of 106 cells/ml. Subsequently, 2 µl fluorescein isothiocyanate-Annexin V (cat. no. 640906; BioLegend, Inc.) and 2 µl 7-aminoactinomycin D (7-AAD; cat. no. 420401; BioLegend, Inc.) were added and incubated for 15 min at room temperature (25°C) in the dark. FACS Calibur (cat. no. 342975; BD Biosciences, Franklin Lakes, NJ, USA) was used to flow cytometric analysis.

Cell proliferation assay. Prior to transfection, HepG2 cells were implanted into 96-well plates at a density of 5,000 cells/well. Cell Counting Kit-8 (CCK-8) solution (10 µl; cat. no. E6465; Dijindo Molecular Technologies, Inc., Kumamoto, Japan) was added to each well, which contained 200 µl culture medium, 48 h post-transfection. After 40 min of incubation at 37°C, optical density values were read at 450 nm.

Caspase-3/-7 activity. Cells were seeded in 96-well plates at a density of 10,000 cells/well (100 µl DMEM with 10% FBS) 24 h before transfection. Caspase-Glo 3/7 reagent (100 µl; cat. no. G8092; Promega Corporation, Madison, WI, USA) was added to each well 48 h post-transfection. A plate shaker was used to lyse cells at 15 x g and room temperature for 1 h. The luminescence of each sample was measured using a plate-reading luminometer (TURNER 9000-001; Promega Corporation), according to the manufacturer’s instructions.

Statistical analysis. All experiments were repeated at least three times. Two groups were compared using the paired Student’s t-test. Multiple groups were compared by two-way analysis of variance followed by Tukey’s post hoc test. All data were expressed as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

NS5A inhibits apoptosis under starvation. pNS5A or control vector was transiently transfected into HepG2 cells, and Annexin V/7-AAD staining was used to determine the number of cells undergoing apoptotic or necrotic processes under different stress conditions by flow cytometry, in order to examine whether NS5A functioned in starvation-induced apoptosis (Fig. 1A). The early, late and total apoptotic cells were quantified and data were displayed as the percentage of apoptotic cells. It was found that NS5A transfection significantly repressed HepG2 cell apoptosis under starvation, compared with the control group under starvation for 24 and 48 h (Fig. 1B and C). However, how NS5A repressed the apoptosis induced by starvation was unclear. Therefore, the protein expression of p53, Bax and cleaved caspase-3 was detected by western blotting (Fig. 1D). Compared with
the control group, transfection with the pNS5A plasmid for 48 h significantly decreased the total p53 and Bax expression levels (Fig. 1D). Furthermore, the conversion of LC3-I to LC3-II was detected, as well as decreased p62 expression compared with the control group, indicating that NS5A may have induced autophagy (Fig. 1D). Caspase-3/-7 activity was attenuated in the pNS5A-transfected group, compared with the control (Fig. 1E), which was consistent with the flow cytometry results under starvation for 24 h. Furthermore, HepG2 cell viability increased in the pNS5A-transfected group, compared with the control group, as detected by CCK-8 assays (Fig. 1F).

Inhibition of autophagy increased the apoptosis of hepatoblastoma cells during starvation. A previous study reported that NS5A protein expression upregulates the mRNA and protein expression of Beclin 1, and also increases Beclin 1 promoter activity by upregulating the NS5ATP9 expression in HepG2 cells (15). The autophagy inhibitors 3-MA and CQ were used to inhibit autophagy to examine whether NS5A-induced autophagy had a role in cell survival or death. HepG2 cell death was detected following incubation with EBSS for 24 h. The present study also detected cell apoptosis under starvation for 48 h. HepG2 cell resistance to 3-MA or CQ was diminished by the longer starvation period (48 h). Therefore, 24 h was selected. Prior studies have shown that 3-MA or CQ inhibit autophagic activity (16). The present study demonstrated that HepG2 cell apoptosis increased in the 3-MA- or CQ-treated groups (Fig. 2A). The quantified results showed that the apoptotic cells increased compared with the control under starvation for 24 h (Fig. 2B). In addition, caspase-3/-7 activity was enhanced in 3-MA- or CQ-treated groups, which was consistent with the flow cytometry results under starvation for 24 h (Fig. 2C). In addition, cell viability increased in the 3-MA- or CQ-treated groups (Fig. 2D).
NS5A-mediated apoptosis inhibition is weakened by autophagy inhibitors 3-MA or CQ. 3-MA and CQ were used to inhibit autophagy to further analyze whether this process was involved in NS5A-mediated apoptosis. Compared with the control group, apoptosis was increased in NS5A-transfected cells treated with 3-MA or CQ (Fig. 3A and B). The quantified results revealed that the number of apoptotic cells treated with 3-MA or CQ increased compared with the control under starvation for 24 h (Fig. 3A and B). This indicated that autophagy was involved and protected cells from apoptosis. Although NS5A reduced apoptosis, this protective effect was eliminated by autophagy inhibition. Furthermore, caspase-3/-7 activity increased in the 3-MA- or CQ-treated groups transfected with NS5A plasmid compared with the control groups (Fig. 3C). This result was consistent with flow cytometry results. In addition, cell viability was decreased in the 3-MA- or CQ-treated groups compared with the control groups (Fig. 3D and E). These results showed that autophagic inhibition may prevent the protective effects of NS5A under starvation.

Apolipoprotein mediated by NS5A is attenuated by Beclin 1 siRNA. Beclin 1 has a central role in regulating autophagy and apoptosis for cellular protection (17). The present study used siRNA and Beclin 1 plasmid to silence and overexpress Beclin 1, respectively, to further confirm the role of Beclin 1 in autophagy and NS5A-mediated apoptosis. Relative caspase-3/-7 activity decreased in the Beclin 1-overexpressed group (Fig. 4A), and significantly increased in the siRNA group (Fig. 4B), compared with the controls.

HepG2 cells were transfected with either control or Beclin 1 siRNA for 24 h, followed by transfection with pNS5A or control for 48 h and subsequent starvation for 24 or 48 h. Apoptosis was determined by caspase-3/-7 activity (Fig. 4C) and flow cytometry (Fig. 4D). The percentage of early, late, and total apoptotic cells was quantified. Compared with the pNS5A transfected group without Beclin 1 siRNA transfection, apoptosis was increased in the pNS5A and Beclin 1 siRNA-transfected group under starvation for 48 h (Fig. 4E and F). The results revealed that Beclin 1 gene silencing attenuated the NS5A-mediated reduction in apoptosis,
demonstrating that Beclin 1 served a role in apoptosis inhibition. Furthermore, the viability of NS5A-transfected cells decreased when Beclin 1 was silenced (Fig. 4G).

**Figure 3.** NS5A-mediated apoptosis inhibition is weakened by autophagy inhibitors 3-MA or CQ. HepG2 cells were transfected with NS5A or control plasmid and starved for 24 h, with or without 3-MA (10 mM) or CQ (10 mM) treatment. (A) Annexin V-7-AAD staining of cells, detected by flow-cytometry in cells treated with 3-MA or (B) CQ; (C) Caspase-3/7 activity was decreased in cells transfected with NS5A. (D) Cell viability was significantly reduced in the 3-MA or (E) CQ treatment groups. Cell viability was determined by Cell Counting Kit-8 assays. Data are presented as the mean ± standard error of the mean. n=3. *P<0.05, **P<0.01, ***P<0.001. NS5A, nonstructural protein 5A; EBSS, Earle's Balanced Salt Solution; 7-AAD, 7-aminoactinomycin D; CQ, chloroquine; 3-MA, 3-methyladenine.

**Beclin 1 mediates the inhibition of apoptosis by NS5A in HepG2 cells.** Beclin 1 is expressed in liver tumor tissues and cancer cell lines, and cannot be detected in healthy liver tissues (6). In addition, HCV infection transcriptionally upregulates the expression of Beclin 1 (18). The increased expression of Beclin 1 may therefore have an important role in starvation-induced cell apoptosis, or Beclin 1-mediated autophagy may be involved in liver cancer induced by HCV infection.

siRNA was used to knockdown Beclin 1 by transfection into HepG2 cells. The western blot results showed that Beclin 1 siRNA effectively inhibited its expression in HepG2 cells, compared with the siRNA negative control (Fig. 5A and B). In addition, the ratio of LC3-I/LC3-II was significantly decreased as a result of the Beclin 1 siRNA transfection (Fig. 5A), whereas it was increased by Beclin 1 plasmid transfection (Fig. 5B). The protein expression of p53, Bax and cleaved caspase-3 in HepG2 cells under starvation for 24 h was also detected by western blotting. Compared with the control group, transfection with Beclin 1 siRNA significantly increased total p53, Bax, and cleaved caspase-3 expression (Fig. 5A), whereas
Beclin 1 plasmid transfection had the opposite effect (Fig. 5B). Next, NSSA plasmid was transfected into the HepG2 cells with Beclin 1 gene silencing to identify the importance of Beclin 1 in NSSA-inhibited apoptosis.

Compared with the pNS5A transfected group without si-Beclin 1, p53, Bax and cleaved caspase-3 protein expression increased in the pNS5A-transfected Beclin 1 siRNA group under starvation for 48 h, compared with the pNS5A-transfected negative control siRNA group (Fig. 5C). These results suggested that the inhibitory effect of Beclin 1 siRNA on autophagy recovered the sensitivity of hepatoblastoma cells to starvation. Therefore, autophagy induced by NSSA may have facilitated the tolerance of hepatoblastoma cells to starvation in a Beclin 1-dependent manner.

**Discussion**

Apoptosis is a critical process in liver cancer. In chronic HCV, downregulation of apoptosis and enhanced cell proliferation not only causes HCV infection persistency in the majority of patients, but also promotes liver cancer (19). It has been reported that several components of HCV influence hepatocarcinogenesis, including NSSA, the envelope protein E2 and the core protein (20,21). NSSA may serve an essential role in HCV-associated liver cancer development by inhibiting cell apoptosis (22). The present study demonstrated that NSSA overexpression inhibited the apoptosis induced by starvation, and enhanced cell viability. HCV NSSA abrogates p53-mediated apoptosis in Hep3B cells by...
suppressing the transcriptional transactivation activity of p53 in a dose-dependent manner (2). The results of the present study revealed that p53, Bax, and cleaved caspase-3 protein expression was decreased in NS5A-expressing HepG2 cells. Our previous studies showed that NS5A induces autophagy in a Beclin 1-dependent manner (15) and autophagy...
mediates the proliferation of HepG2 cells (16). Autophagy, which provides nutrients by degrading existing cellular components, is considered an adaptive response to various cellular pressures, including hypoxia and starvation (23,24). It has been speculated that the effects of autophagy on cell survival and apoptosis may vary depending on the cell type, micro-environment, and the extent of autophagy induction (25). Autophagy in tumor therapy is associated with chemotherapy, radiation and immune tolerance (25,26). However, autophagy is also an indispensable physiological response required to maintain the viability of cells during starvation (27). In addition, increased autophagy following treatment with antitumor drugs reduces their effects (28). Furthermore, inhibition of autophagy in starved HeLa cells promotes apoptosis and caspase-3 activation (29,30). The present study demonstrated that the inhibition of autophagy by 3-MA or CQ significantly increased the apoptosis of starved cells. Further, Beclin 1 expression was also silenced to inhibit autophagy, which also increased HepG2 cell apoptosis. Therefore, it was speculated that Beclin 1-dependent autophagy may have mediated apoptosis inhibition by NS5A.

Beclin 1 is a major regulator of autophagy (31). In a previous study, the results of a cDNA microarray screen and northern blot analysis demonstrated that the Beclin 1 gene expression is upregulated in liver cancer tissues (11). It has also been shown that Beclin 1 serves a major role in liver cancer. The overexpression of Beclin 1 increases cell survival by inhibiting apoptosis (32,33), and its silencing results in the increased sensitivity of cells to stress (34). Furthermore, miR-216a enhances the radiosensitivity of pancreatic cancer cells by suppressing Beclin 1-mediated autophagy (35). The combined delivery of Beclin 1 siRNA and FTY720 (a novel immunosuppressive agent with effective anticancer properties) more efficiently inhibits liver cancer cell progression by suppressing protective autophagy and increasing apoptosis (36), which is consistent with the results of the present study. The present study found that Beclin 1 inhibited p53, Bax, and cleaved caspase-3 expression in starved HepG2 cells. Furthermore, it was confirmed that Beclin 1 expression by NS5A was involved in the negative regulation of starvation-induced liver cancer apoptosis, which was accompanied by reduced p53 and apoptosis regulator Bax expression, as well as decreased caspase-3/-7 activation. Combining siRNA and anticancer drugs into the same vector has unique advantages (36). Therefore, it was speculated that the combined treatment of Beclin 1 siRNA with anticancer drugs may effectively promote the drug’s efficacy and significantly improve HCC treatment.

In conclusion, HCV NS5A inhibited starvation-induced apoptosis by downregulating p53-associated signaling pathways, and this process was mediated by upregulating Beclin 1-dependent autophagy in human hepatoblastoma cells. This suggested that Beclin 1 was vital in starvation-induced apoptosis and mediated NS5A-inhibited apoptosis. However, this was not the only mechanism by which NS5A inhibited the apoptosis of liver cancer cells. Even following autophagy inhibition or Beclin 1 silencing, the NS5A-induced effects of HepG2 cell apoptosis inhibition and proliferation increase were blocked. However, the inhibition was significant and thus may provide treatment clues for HCV-associated liver cancer. Recently, the combination of siRNA and anticancer drugs into the same vector has been shown to downregulate the expression of cancer-associated genes and promote the effect of cancer drugs on tumor sites to effectively inhibit tumor progression (37,38). Therefore, the results of the present study may aid in developing a novel therapeutic strategy to prevent cancer cells from adapting to stress. Inhibition of autophagy or Beclin 1 targeted siRNA may be promising strategy for use in HCV-associated liver cancer therapy. Therefore, in view of Beclin 1 and its role in autophagy, it is likely that Beclin 1 targeting in the treatment of HCV-associated liver cancer may have better therapeutic effect. However, NS5A-mediated hepatocarcinogenesis is only one part of HCV-mediated tumorigenesis. Therefore, the exact mechanism of HCV-associated liver tumor remains to be elucidated. Further animal experiments research is needed to verify the experimental results of the present study.

Acknowledgements

Not applicable.

Funding

This study was funded by Beijing Key Laboratory of Emerging Infectious Diseases Project (grant no. DTKF201704), the National Natural Science Foundation of China (grant no. 81470863), and the Beijing Municipal Administration of Hospitals' Ascent Plan (grant no. DFL20151701).

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

MQ and JC designed the experiments. MQ wrote and revised the manuscript. SF and SL performed the flow cytometric analysis. LZ and YZ cultured the HepG2 cells and conducted the transfection experiment. SF and SL performed the caspase-3/-7 activity assay and cell proliferation detection. MQ performed plasmid construction and western blotting. JC supervised all experiments and analyses. All authors read and approved the manuscript, and agreed to be accountable for all aspects of the work.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
References

5. Peng L, Liang D, Tong W, Li J and Yuan Z: Hepatitis C virus NS5A activates the mammalian target of rapamycin (mTOR) pathway, contributing to cell survival by disrupting the interaction between FK506-binding protein 38 (FKBP38) and mTOR. J Biol Chem 285: 20870-20881, 2010.